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Inhibition of *Listeria monocytogenes* by bovicin HC5, a bacteriocin produced by *Streptococcus bovis* HC5☆

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Abstract

Cattle can be infected with *Listeria monocytogenes* by consuming contaminated plant materials, soil or silage, and farmers have sought ways of preventing this contamination. Recent work indicated that *Streptococcus bovis* HC5 produced a bacteriocin (bovicin HC5) that could inhibit a variety of gram-positive bacteria, and we examined the ability of bovicin HC5 to inhibit 10 strains of *L. monocytogenes* that had been isolated from plant materials, soil, silage and infected cattle. Growth experiments indicated that all of the *L. monocytogenes* strains were inhibited by 100 activity units (AU) of bovicin HC5 ml^{-1}. *L. monocytogenes* cultures that were transferred with sublethal doses (12.5 AU ml^{-1}) could be adapted in stepwise fashion to higher doses of bovicin HC5. However, even 'adapted' cultures did not grow if 400 AU ml^{-1} was added. The effect of bovicin HC5 on *L. monocytogenes* was bactericidal, and viability decreased 5–7 logs after only 2 h of exposure. Bovicin HC5 caused a nearly complete efflux of intracellular potassium in 15 min but only if the pH was less than 6.0. When the pH was greater than 6.0, the cells maintained their potassium pool. *L. monocytogenes* cells that were acid-adapted (final pH of 4.6) were as sensitive to bovicin HC5 as those that were not acid-adapted (final pH of 6.3). These results support the idea that bovicin HC5 could be effective in controlling listeria in contaminated silages.
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Keywords: *Listeria monocytogenes*; Bacteriocins; *Streptococcus bovis*; Silage

1. Introduction

Listeria monocytogenes causes more than 2500 cases of food-borne illnesses in the United States each year, and cattle can also be infected (Mead et al., 1999). When infected cattle shed listeria in their feces, fruits, vegetables and milk products can be contaminated (Jay, 1996), and this latter type of contamination is a particular problem for the soft cheese industry (Rudol and Scherer, 2001). Listeria are rapidly growing, lactic acid-producing bacteria that are nearly as pH-resistant as lactobacilli (Tienungoon et al., 2000), and listeriosis in cattle is typically caused by contaminated silage (Stark and Wilkinson, 1988; Wiedemann et al., 1996). Listeria can grow at low temperatures (Tienungoon et al., 2000), and hay crop

☆ Proprietary or brand names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product, and exclusion of others that may be suitable.

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silage stored in large plastic bags has frequently been contaminated (Wilkinson, 1999).

Silage fermentation is promoted by a variety of lactic acid bacteria, but the naturally occurring microbiota is sometimes insufficient to prevent the growth of detrimental bacteria such as clostridia and *L. monocytogenes* (McDonald et al., 1991). When Jones et al. (1991) examined the ability of bacteria to serve as silage inoculants, they noted that *Streptococcus bovis* "grew faster than any of the commercial species tested and resulted in the most homolactic fermentation and lowest ammonia concentration." These latter authors did not indicate if their *S. bovis* inocula produced bacteriocins, but recent work indicated that *S. bovis* HC5 produced a bacteriocin (bovicin HC5) that could kill at least one strain of *L. monocytogenes* (1043S) (Mantovani et al., 2002).

L. monocytogenes is sensitive to a variety of bacteriocins (e.g. nisin, pediocin, lactococcin, etc.). However, this bacterium can become highly bacteriocin-resistant and retain this phenotype even if the bacteriocin is not present (Rekhif et al., 1994; Gravesen et al., 2002). Rekhif et al. (1994) hypothesized that the bacteriocin resistance of *L. monocytogenes* was mediated by a high-frequency mutation (as high as 10^{-4}). Mazzotta and Montville (1997) noted that nisin-resistant *L. monocytogenes* cells had more C-16 and C-18 fatty acids in their cell membranes than nisin-sensitive strains, but the role of these acids in preventing nisin from disrupting membrane gradients was not clearly defined.

We examined the ability of bovicin HC5 to inhibit 10 strains of *L. monocytogenes* that had been isolated from plant materials, soil, silage and infected cattle. The strains were transferred with sublethal doses of bovicin HC5 to monitor the development of bacteriocin resistance.

2. Materials and methods

2.1. Cell growth and media

S. bovis HC5 and methods of culture were previously described (Mantovani et al., 2001). The *L. monocytogenes* strains (provided by Martin Wiedemann, Department of Food Science, Cornell University) were isolated from plant materials, soil, contaminated silage and infected cattle (see Table 1). The ribotyping was described by Wiedemann et al. (1996). *L. monocytogenes* was grown anaerobically and statically in BHI (brain heart infusion) medium (Becton, Dickinson & CO., Sparks, MD, USA) that had an initial pH of 7.4 ± 0.2, and the final pH was typically 5.8. If the initial glucose concentration was increased from 10 to 30 mM, the final pH was always 4.6 ± 0.1. When BHI was supplemented with phosphate (equal parts mono and dibasic sodium phosphate, 100 mM total, pH 7.0), the final pH was never less than 6.3.

2.2. Agar overlays

Initial screens of bacteriocin activity were based on a procedure that employed an agar overlay. *S. bovis* HC5 was spotted onto anaerobic agar plates that contained a carbonate-buffered medium (Mantovani et al., 2001). After 24 h of incubation at 39 °C in an anaerobic glove box (Coy Laboratory Products, Ann Arbor, MI), the plates were removed from the glove box and overlayed with soft BHI agar (0.7% agar, approximately 10^6 actively growing *L. monocytogenes* cells). The plates were incubated aerobically at 4 °C (a temperature that does not allow the growth of *S. bovis* HC5) for 6 h. The agar overlay was then incubated aerobically at 37 °C for 24 h to assess the activity of *S. bovis* HC5 against each strain of *L. monocytogenes*.

2.3. Preparation and activity of bovicin HC5

Bovicin HC5 was liberated from the cells via a method that employed acidic NaCl (Yang et al., 1992;

Table 1
Sensitivity of *L. monocytogenes* strains to *S. bovis* HC5

Strain	Isolated from	Ribotype	Sensitivity to *S. bovis* HC5*
CU-SIDII-7	silage	Dup-1045	18.7 ± 1.8
CU-SIK17/93	silage	Dup-1030	17.0 ± 1.4
CU-SI163/94	silage	Dup-1030	16.5 ± 0.7
DL 773014	bovine	Dup-1038	17.5 ± 1.0
EI-146	plant	Dup-1039C	17.0 ± 2.6
EI-203	plant	Dup-1045A	18.0 ± 1.4
EI-204	plant	Dup-1045A	15.3 ± 1.8
EI-250	plant	Dup-1023	17.0 ± 0.5
EI-266	soil	Dup-1042B	17.0 ± 1.5
EI-271	plant	Dup-1053	17.1 ± 2.2

* Inhibition zone (mm).

Mantovani et al., 2002). Stationary phase *S. bovis* HC5 cultures (1 l, approximately 400 μg ml^{-1}) were heated to 70 °C for 30 min. The cells were harvested by centrifugation (8000 × *g*, 15 min, 5 °C), and the culture supernatant was discarded. The cell pellets were washed in sodium phosphate buffer (50 ml, 5 mM, pH 6.7) and resuspended in acidic sodium chloride (100 mM, pH 2.0, 4 °C, 2 h). The cell suspensions were then recentrifuged (8000 × *g*, 15 min, 5 °C), and the cell-free supernatant was lyophilized (Lyph-Lock 4.5 lyophilizer, Labconco, Kansas City, MO). The lyophilized material was resuspended in sterile distilled water (2 ml). The preparation was assayed for antibacterial activity by serially diluting the extract (20 μl) in distilled water (twofold increments), and placing each dilution (10 μl) on a lawn of *L. monocytogenes* CU-SI 163/94 that had been grown anaerobically in BHI agar with an inoculum of approximately 10^6 cfu ml^{-1} (39 °C). Bovicin HC5 activity units (expressed per millimeter) were calculated from the reciprocal of the highest serial dilution showing a visible zone of clearing (Lee et al., 2002).

2.4. Acid adaptation

L. monocytogenes cultures were acid-adapted by adjusting the initial pH of the BHI broth. Cultures grown in BHI broth had a final pH of approximately 5.8, but the final pH was 4.6 if the initial pH was adjusted to 5.5 with HCl.

2.5. Potassium efflux

Stationary cultures of *L. monocytogenes* CU-SI 163/94 were harvested by centrifugation (10,000 × *g*, 10 min, 5 °C), and the cell pellets were washed, resuspended and energized in MES buffer (100 mM 2-[*N*-morpholino] ethanesulfonic acid, 10 mM $MgSO_4 \cdot 7H_2O$, 10 mM KCl, pH 7.0, 30 °C, 10 min, 20 mM glucose). The pH of cell suspensions (200 μg protein ml^{-1}) was adjusted in stepwise fashion from 7.0 to 5.0 with HCl. The washed cell suspensions were energized with glucose (20 mM, 10 min, 39 °C) and treated with bovicin HC5 (640 AU ml^{-1}, 30 °C, 30 min). The cell suspensions (1.0 ml) were centrifuged through silicone oil (0.3 ml Dow Corning 550 fluid, Dow Corning, Midland, MI) to quickly separate the cells from culture medium (Mantovani et al., 2002). The tubes were then frozen (−15 °C, 1 h). Once the liquid above the silicone solidified, cell pellets were removed with dog nail clippers and digested in HNO_3 (3 N, 22 °C, 24 h, Mantovani et al., 2002). Insoluble cell debris was removed by centrifugation (13,000 × *g*, 22 °C, 5 min). Potassium in the nitric acid digests was determined by flame photometry (1, 2, 3 and 5 μg ml^{-1} potassium standards, Cole-Parmer 2655-00 Digital Flame Analyzer, Cole-Parmer Instrument, Chicago, IL).

2.6. Other analysis

Bacterial growth was monitored via changes in optical density (3-ml sample, 1-cm cuvette, 600 nm, Gilford 260 spectrophotometer, Oberlin, OH), and the ratio of total cell protein to optical density was approximately 200 μg protein ml^{-1} optical density $unit^{-1}$. Total cell protein from NaOH-hydrolyzed cells (0.2 M NaOH, 100 °C, 15 min) was determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

2.7. Experimental design and statistical analysis

All incubations and determinations were performed two or more times, the results were highly reproducible, and the coefficients of variation (standard deviations ÷ means) were always less than 10%. When error bars are given in the table or figures, they refer to the standard deviation.

3. Results

3.1. Bovicin HC5 activity

Preliminary experiments indicated that *S. bovis* HC5 inhibited the growth of 10 *L. monocytogenes* strains in agar overlays, and colonies were never observed in the zones of clearing (Fig. 1, Table 1). Growth could also be inhibited by a bovicin HC5 extract (Fig. 2). When the bovicin HC5 extract was serially diluted (twofold increments) and spotted (10 μl) onto the surface of a BHI plates spread with a lawn of *L. monocytogenes* CU-SI163/94, zones of clearing were observed until the dilutions were 256-fold.

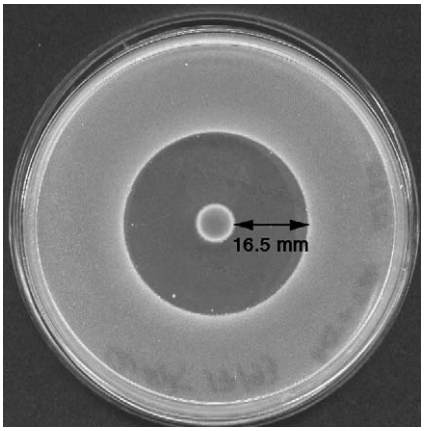

Fig. 1. An agar overlay showing the inhibition of *L. monocytogenes* (strain CU-SI 163/94) by *S. bovis* HC5. *S. bovis* HC5 was spotted (5 μl) on a basal agar medium and incubated anaerobically (39 °C, 24 h). The plate was then overlayed with molten agar containing *L. monocytogenes* (approximately 10^6 cells ml^{-1}).

Based on this dilution and extract volume (10 μl), the extract had approximately 25,600 activity units (AU) ml^{-1}.

3.2. Adaptation of L. monocytogenes to bovicin HC5

When the 10 strains of *L. monocytogenes* were inoculated into BHI broth, growth was detected until the concentration of bovicin HC5 was approximately 100 AU ml^{-1} and the strain variation was less than twofold (Fig. 2a). By adjusting the initial pH with HCl, it was possible to decrease the final pH to 4.6. The acid-adapted *L. monocytogenes* were no more resistant to bovicin HC5 (Fig. 2b) than cultures that had not been adapted to acid (Fig. 2a). If the cultures were transferred in stepwise fashion with increasing concentrations of bovicin HC5, the increase in resistance was fourfold or less even if the pH was decreased from 7.4 to 5.5. Cultures that were transferred repeatedly with 100 AU of bovicin HC5 ml^{-1} never completely adapted and grew very slowly.

3.3. Mode of action

L. monocytogenes was inhibited by bovicin HC5, but these growth experiments did not indicate if the activity was bactericidal or merely bacteriostatic. To differentiate these effects, the most resistant strain (CU-SI163/94) was grown in BHI without bacteriocin, and stationary-phase cells (200 μg ml^{-1}) were suspended in sodium phosphate buffer that had 640 AU ml^{-1} of bacteriocin. Samples were then withdrawn and diluted (tenfold increments) into BHI broth to determine viability (37 °C, 24 h of incubation). Results indicated that cells exposed to bacteriocin for

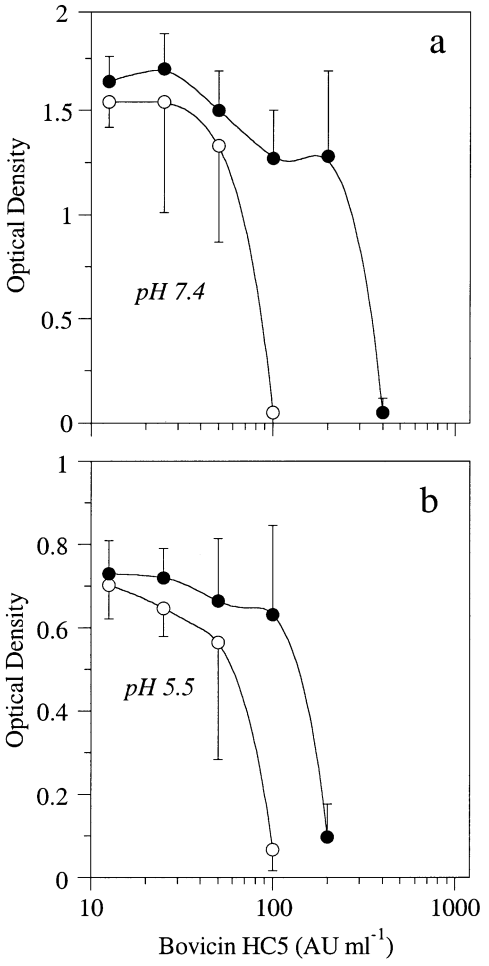

Fig. 2. The effect of bovicin HC5 on the growth of 10 strains of *L. monocytogenes*. Part (a) shows an initial pH of 7.4 and part (b) shows growth when the initial pH was 5.5. Open circles show the growth of nonadapted cultures. The closed circles show the growth of 'adapted' cultures that were successively transferred into higher concentrations of the bacteriocin. Error bars indicate standard deviations ($n = 10$ different strains).

as little as 2 h at pH 6.5 or 5.5 had a very low viability (Fig. 3).

L. monocytogenes CU-SI163/94 cells that were washed and incubated in MES buffer containing glucose (20 mM) had an intracellular potassium content of approximately 1500 nmol mg protein^{-1} at pH values ranging from 7.0 to 5.0, and the potassium content did not decrease for more than 30 min. Bovicin HC5 (640 AU ml^{-1}) caused a decrease in potassium, but only if the pH was less than 6.0. If the pH was greater than 6.0, the cells maintained their potassium for at least 30 min (Fig. 4).

3.4. Effect of induced acid adaptation on bacteriocin resistance

L. monocytogenes cultures that were inoculated into BHI containing 30 mM glucose had a final pH of 4.6, but the final pH was 6.3 if the glucose was decreased to 10 mM and sodium phosphate (100 mM, equal parts di and mono basic) was added to the medium (Fig. 5). Experiments with washed cell suspensions (20 mM glucose, MES buffer, pH 5.5) indicated that both cell types had approximately the same sensitivity to bovicin HC5.

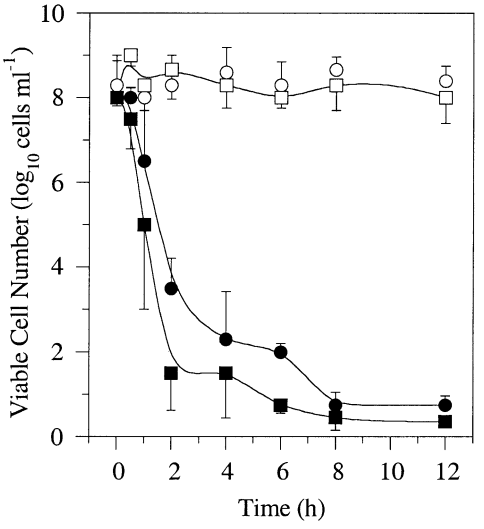

Fig. 3. The viability of washed cell suspensions (approximately 200 μg ml^{-1}) of *L. monocytogenes* CU-SI 163/94 that were exposed to bovicin HC5 (640 AU ml^{-1}) for varying amounts of time. Closed circles show pH 6.5 and closed squares show pH 5.5. Open symbols show untreated controls. Error bars indicate standard deviations ($n=3$).

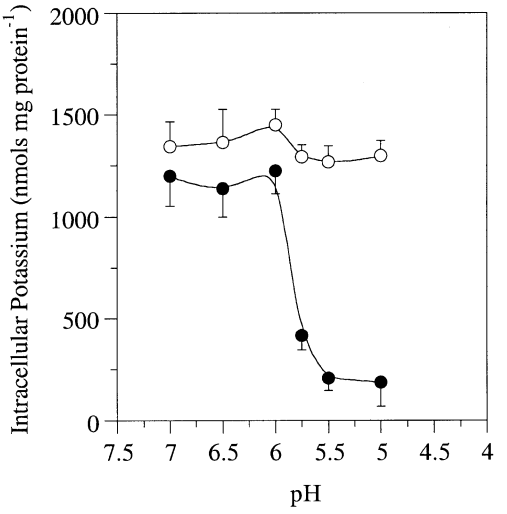

Fig. 4. The effect of pH on the intracellular potassium content of glycolyzing *L. monocytogenes* CU-SI 163/94 cell suspensions that were treated with bovicin HC5 (640 AU ml^{-1}, 30 °C, 30 min) (closed circles). Untreated controls are also shown (open circles). Error bars indicate standard deviations ($n=3$).

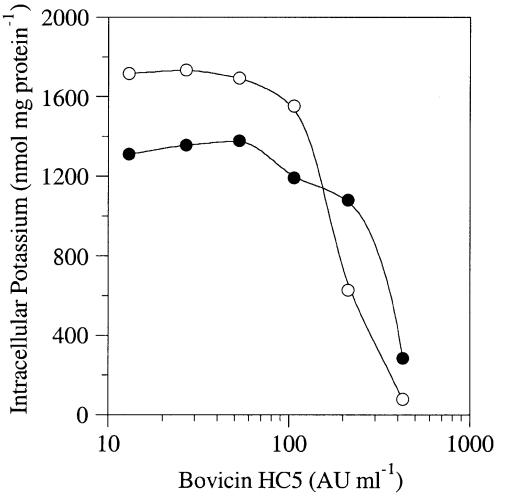

Fig. 5. The effect of bovicin HC5 on the intracellular potassium of glycolyzing *L. monocytogenes* CU-SI 163/94 cells that had been grown in BHI when the final pH was 6.3 (open circles). The closed symbols show cells that had been grown in BHI when the final pH was 4.6.

4. Discussion

Previous work indicated that wild-type *S. bovis* strains were inhibited by nisin, but nisin-resistant cells were detected after only a few hours of incubation (Mantovani and Russell, 2001). Nisin-resistant *S. bovis* cells had more lipoteichoic acids than nisin-sensitive strains, and the cells were more positively charged. Because nisin is a positively charged molecule, it appeared that resistant cells were excluding nisin (Mantovani and Russell, 2001). The nisin-resistant *S. bovis* cultures were also resistant to bovicin 255 of *Streptococcus gallolyticus*. Subsequent work indicated that *S. bovis* HC5 produced a bacteriocin (bovicin HC5) that inhibited nisin-resistant as well as nisin-sensitive *S. bovis* cells and adaptation was not observed (Mantovani et al., 2001). Based on these results, it appeared that bovicin HC5 might be a more potent and useful bacteriocin than nisin.

Some bacteriocins are only bacteriostatic (Morovsky et al., 1998; Atrih et al., 2001; Moreno et al., 2002), but the action of bovicin HC5 against *L. monocytogenes* was bactericidal. Large declines in viability were observed even if the cells were exposed to bovicin HC5 for only 2 h at pH 5.5 or 6.5. Okereke and Thompson (1996) and van Schaik et al. (1999) noted that acid-adapted *L. monocytogenes* were more resistant to nisin than cells that had been grown at neutral pH. However, our experiments indicated that the pH of the growth medium had little impact on the sensitivity of *L. monocytogenes* to bovicin HC5. These results supported the idea that *L. monocytogenes* could not adapt and become highly bovicin HC5-resistant.

Bacteriocin activity is typically monitored by decreases in viability, but these effects alone provide little information regarding the mode of action. Previous work indicated that bovicin HC5 was a pore-forming bacteriocin that catalyzed potassium efflux from *S. bovis* JB1 (Mantovani et al., 2002), and potassium efflux from *L. monocytogenes* CU-SI 163/94 was also observed. The effect of bovicin HC5 on *S. bovis* JB1 is greater when the pH is acidic (data not shown), and similar results were noted with *L. monocytogenes* CU-SI 163/94. Little potassium depletion was observed when the pH was greater than 6.0, but cells could not maintain their potassium for 30 min if the pH was less than 6.0.

Kalmokoff et al. (1996) proposed the idea that bacteriocin-producing bacteria might be useful in silage fermentation, and Whitford et al. (2001) purified, cloned and sequenced a bacteriocin from *S. gallolyticus* LRC0255 (bovicin 255). Bovicin 255 had some activity against listeria, but many strains of *S. bovis* adapted to bovicin 255 and became highly resistant (Mantovani et al., 2001). The present work indicates that *L. monocytogenes* did not adapt to bovicin HC5 and this observation supports its potential application as a silage inoculant.

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